

Biocompatibility of two commercial forms of mineral trioxide aggregate

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Abstract

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Aim To examine the biocompatibility of two commercial forms of mineral trioxide aggregate (MTA), by evaluating the morphology of an established cell line.

Methodology The two cements were cast on glass cover slips and cured for 1 or 28 days. Saos-2 osteosarcoma cells were trypsinized and seeded at a density of 1×10^5 cells and were then placed in medium over the material-coated coverslips for 1, 5 and 7 days. After these time intervals the media were discarded and the cells fixed. Cell morphological investigation was performed by scanning electron microscopy at various magnifications ranging from $\times 250$ to

$\times 500$. The biocompatibility of cement constituents, alusilicate flux and bismuth oxide was also investigated.

Results All cement samples cured for 1 day showed a confluent cell monolayer after 5 and 7 days. The response to both materials was similar. Materials cured for 28 days showed incomplete cell confluence after 1 and 5 days. Alusilicate flux and bismuth oxide did not demonstrate biocompatibility.

Conclusions The 1-day cured samples of two commercial forms of MTA showed good biocompatibility. However, the 28-day cured samples were less biocompatible after 1 and 5 days.

Keywords: biocompatibility, mineral trioxide aggregate, cell culture, scanning electron microscopy, critical-point drying.

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Introduction

In the 1990s a new material, mineral trioxide aggregate (MTA) was developed as a root-end filling material. It was investigated because it is a hydraulic cement that sets in the presence of water, an important property for any dental cement. It is commercially available as ProRoot MTA (Tulsa Dental Products, Tulsa, OK, USA).

The biocompatibility of the original form of MTA (which was grey in colour) has been investigated using cell culture techniques with established human osteo-

sarcoma cells (Koh *et al.* 1997, 1998, Mitchell *et al.* 1999, Abdullah *et al.* 2002). These Saos-2 cells represent a highly differentiated cell line capable of inducing bone formation and therefore create a model for studying bone cell behaviour. Recently a new tooth-coloured form of MTA has been marketed. There has only been one investigation, and that was conducted on its biocompatibility (Perez *et al.* 2003). The reason for the biocompatibility of MTA has so far not been clearly established. MTA is based on Portland cement; both grey and white Portland cements are manufactured from similar raw materials except that a fluxing agent is used for production of the white version to remove the ferrite phase during the clinking process (Glasser 1983). Bismuth oxide is added to MTA for radiopacity.

The aim of this study was to examine the biocompatibility of two commercial forms of MTA, and to

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investigate the biocompatibility of the cement constituents, alusilicate flux and bismuth oxide, using a cell culture technique.

Materials and methods

Surface morphology of cements and powders

For each form of MTA (grey and white) (ProRoot MTA) 1 g was mixed with 0.35 mL of distilled water to produce a homogeneous paste. The materials were compacted in brass moulds 5 mm in diameter and left to cure in the moulds at 100% humidity for 6 h to allow the cements to gain sufficient strength. After demoulding, the materials were further left to cure at 100% humidity and 37 °C for 1 or 28 days. Two samples were made for each material for examination at each time interval, and were viewed at $\times 250$ – 500 magnification. The materials were then mounted on aluminium stubs (Agar Scientific, Stansted, UK) with carbon cement (Leit-C Emitech, Ashford, UK) and were carbon coated (Emitech K250) for electrical conductivity. The bismuth oxide and alusilicate powders were sprinkled on Perspex mounted on aluminium stubs and then carbon coated. Two samples were made for each material. The materials were viewed under the scanning electron microscope (SEM S3500; Hitachi, Wokingham, UK). Secondary electron imaging was performed for the surface analysis of the materials.

Preparation of test material for cell culture study

To prepare the test materials 0.125 g of white or grey MTA (ProRoot MTA) were mixed on a glass slab according to the manufacturer's instructions. The mixed materials were transferred to borosilicate glass coverslips (thickness No. 1; BDH, Poole, UK), which had been previously lightly sandblasted using Aquacut (Medivance Instruments Ltd, London, UK). The sandblasting provided a rough surface for better adhesion of the materials to the glass. A total of four coverslips were prepared for each material in each group. The coverslips were placed in tissue culture dishes. The materials were cured for 1 or 28 days in an incubator at 37 °C and 100% humidity. The alusilicate and bismuth oxide powder were attached to the glass coverslips with double sided tape and were then placed in the tissue culture dishes.

Cytomorphology of osteoblasts

The human osteosarcoma cell line, Saos-2, was used. Thawed cells were grown in 75 cm³ flasks containing 12 mL Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, penicillin and streptomycin and glutamine and grown until they reached confluence in a humidified incubator at 37 °C with 5% CO₂ in 95% air. At 80% confluence the cells were trypsinized, counted and seeded at a density of 1×10^5 cells per 35 mm dish in 75 mL of media. After 30 min, 3 mL of media was added to each Petri dish.

When cured the material-coated coverslips were subjected to ultraviolet light sterilization for 12 h. An aliquot of 0.75 mL DMEM was placed on the test material on the glass coverslip. Tissue culture dishes without the test materials were used as controls. Two controls were prepared for each group. The tissue culture dishes were kept in an incubator at 37 °C with 5% carbon dioxide in 95% air. After 1, 5 and 7 days the media were removed and the material-coated coverslips were covered with 2.5% glutaraldehyde in cacodylate buffer for 4 h to fix the cells. The glutaraldehyde was removed and the material was washed with sucrose-cacodylate-based buffer (pH 7.3) for 4 h. The material-coated glass coverslips with the fixed cells were prepared for viewing under SEM. The cells were desiccated with ascending grades of ethanol and were then critical-point dried (Emitech K850). The critical-point drying took the specimens to 31.1 °C and 1072 psi. The material-coated coverslips were then mounted on aluminium stubs (Agar Scientific) with carbon cement (Leit-C Emitech) and were coated with 5–7 nm of gold (Emitech K250) for electrical conductivity. The samples were then viewed under the SEM. Semi-quantitative analysis was performed by two observers jointly using a scoring method of both cell quantity and morphology: 0, no cells; 1, occasional round cells; 2, sparse flattened out cells; 3, substantial cell growth mostly flat cells with a few rounded cells; 4, confluence.

Evaluation of specimen preparation

The suitability of the processing technique for testing the biocompatibility of the materials was investigated to ascertain that the material was not being affected. Discs 10 mm in diameter of grey MTA were cast and cured for 7 days. The discs were then placed in tissue culture dishes and cells were seeded and incubated on the

materials for 1 day, after which the media was discarded and samples processed for SEM. The following processing techniques were undertaken:

1. Material fixed with a 2.5% cacodylate-based glutaraldehyde fixative, washed and air dried.
2. Material fixed with a 2.5% cacodylate-based glutaraldehyde fixative, washed and critical-point dried.
3. Material fixed with a 2.5% cacodylate-based glutaraldehyde fixative, washed, treated with hexamethyldisilazane (HMDS) and air-dried.

Two samples were prepared for each processing technique. The processing was carried out with the cells seeded on the materials as well as with the materials alone.

Results

Surface morphology of cements and powders

The surface of MTA cured for 1 day and for 28 days was similar. The surface of the material appeared smooth with no crystalline structures. Both materials appeared to have a similar surface morphology. The alusilicate flux was made up of globular particles varying in size while the bismuth oxide was composed of medium sized granules. The bismuth oxide particles had a tendency to clump together making estimation of particle size difficult.

Cytomorphology of osteoblasts

A total of four coverslips were examined for each material under each condition. Coverslips were collected after 1, 5 and 7 days of cell growth thus a total of 96 samples were viewed. Table 1 shows the scores for the 1-day cured materials. At 1 day the cells that were present were masked by diffuse crystals; therefore no score was given. At 5 days a cell monolayer (Score 4) was visible over both MTA materials (Fig. 1); and at 7 days a cell monolayer could be identified, but some rounded cells were also present (Score 3).

The scores for the 28-day cured materials are shown in Table 2. The samples viewed after 1 day of cell growth showed at best sparse growth for the grey MTA

Table 1 Mean scores ($\pm$ SD) for 1-day cured materials ($n = 4$)

Period of exposure (days)	Grey MTA	White MTA	Alusilicate	Bismuth oxide
1	—	—	0	0
5	4.0 ± 0	3.75 ± 0.5	0	0
7	2.75 ± 0.5	3.0 ± 0.8	0	0

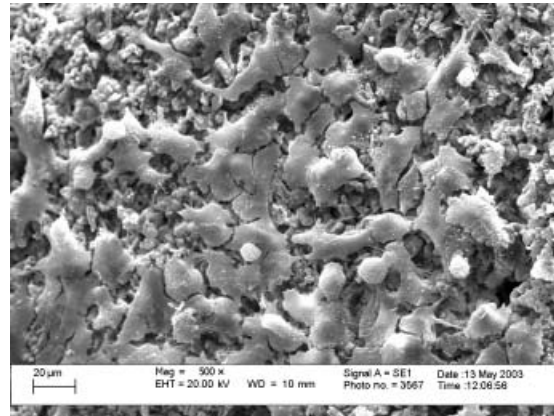


Figure 1 Surface morphology of cells incubated for 5 days on white MTA cured for 1 day (Score 4) (magnification $\times 500$).

Table 2 Mean scores ($\pm$ SD) for 28-day cured materials ($n = 4$)

Period of exposure (days)	Grey MTA	White MTA	Alusilicate	Bismuth oxide
1	1.5 ± 1.0	0.5 ± 0.6	0	0
5	1.25 ± 0.9	1.75 ± 0.9	0	0
7	3.75 ± 0.5	2.75 ± 0.5	0	0

(Score 2; Fig. 2) and occasional cells for the white MTA (Score 1). After incubating the cells for 5 days there was still sparse growth over the materials (Score 2). However, after 7 days, there was an appreciable cell growth with confluence over grey MTA (Score 4) and fewer cells but flat ones for white MTA (Score 3).

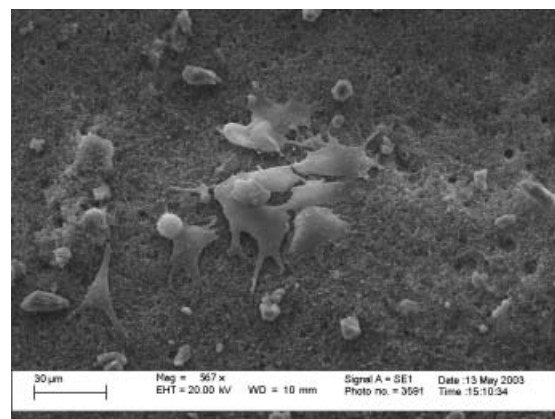


Figure 2 Surface morphology of cells incubated on white MTA cured for 28 days (Score 2) (magnification $\times 567$).

There were no cells over any sample of bismuth oxide and alusilicate. There were confluent cells (Score 4) growing over the glass coverslips, which were not covered by material and over the controls.

Evaluation of specimen preparation

There was no change in material morphology when the material was treated with cacodylate-based fixative washed and air-dried. However, the cells were not well preserved, and showed considerable shrinkage and loss of morphology.

The critical-point dried samples showed a change in the surface morphology of the material. The surfaces showed crystalline deposits, which were diffuse and small, or large. Good cell morphology was observed.

The samples treated with HMDS also showed diffuse crystals over their surfaces but no cells. Only cell remnants were present on the materials.

Discussion

In this study a cell culture technique was employed; it was modified over that used in a previous study (Abdullah *et al.* 2002). The cement materials being investigated produce calcium hydroxide as a byproduct of the hydration reaction (Camilleri *et al.* 2004), and therefore a cacodylate-based fixing solution was used to avoid the media reacting with the calcium hydroxide. The cacodylate-based glutaraldehyde fixing solution had been previously used by Zhu *et al.* (2000), but in that study the cements were washed with phosphate buffered saline prior to fixing thus potentially contaminating the material.

Critical-point drying and ethanol solutions may also affect the material. The calcium hydroxide produced during setting may carbonate on contact with CO₂ to produce a precipitate of calcium carbonate on the specimens; the precipitate may affect the field viewed under the SEM. This was noted particularly in the group where cells had been grown for 1 day on 1-day cured MTA. Thus, critical-point drying cannot be recommended for processing materials based on Portland cement. Other techniques need to be adopted for preserving cells prior to viewing under the SEM.

In the present study the MTA surfaces exposed to the culture medium demonstrated carbonation. In contrast the SEM analysis of cements cured at 100% humidity showed a smooth surface morphology. The samples that were fixed with glutaraldehyde, washed and dehydrated showed carbonation products over the

surface. The presence of moisture is essential to the carbonation process as CO₂ does not react with dry calcium hydroxide (Lea 1970). The carbonation process was accelerated by critical-point drying that utilized free CO₂ at high temperature and pressure to dry the samples. The calcium carbonate formed was crystalline. Various types of crystal shapes were produced; these included diffuse small crystals (Fig. 3a), larger hexagonal crystals (Fig. 3b), globular shapes (Fig. 3c) and coral-like surfaces (Fig. 3d). The diffuse crystals were the most predominant with hexagonal large crystals being present at the material edge. The diffuse crystals could have been calcites, and the hexagonal-shaped crystals, aragonites; these crystals have the same chemical composition but different crystal structures and symmetries. The aragonite polymorph could have been synthesized by the high temperature and pressure produced by critical-point drying. The coral-like appearance had previously been described by Abdullah *et al.* (2002) but not identified. The material could have been calcium carbonate in aragonite form, which may occasionally present in the *flos ferri* form or even hydrating cement grains with calcium silicate hydrate gel fibres around the reacting cement particle. The globular appearance which had previously been described by Torabinejad *et al.* (1995) and Koh *et al.* (1997), but not identified, was most likely to be calcium carbonate in vaterite form. The presence of these carbonation products is considered to be due to the critical-point drying which is a routine procedure used in preparation of biological specimens. Crystals were identified by observing crystal shapes observed on SEM; X-ray analysis of the various crystals forms would be necessary to confirm the exact symmetry and polymorphism.

Phosphate deposition from the fixing solution (Koh *et al.* 1997, 1998, Mitchell *et al.* 1999, Abdullah *et al.* 2002) was avoided by adopting a modified technique utilizing cacodylate-based fixative and washing solutions. In addition, the substitution of critical-point drying was also taken into consideration due to heavy carbonation. Critical-point drying is necessary when processing tissue samples as allowing tissue to dry in air or under vacuum during the metallization process causes damage to the surface that is to be examined in the SEM. Tissue samples become damaged by normal air drying because of very large surface tension forces created in cavities of small dimensions when there is a liquid/gas interface. As tissue dries, the liquid/gas interface travels through the surface of the material collapsing the cavities between projecting structures.

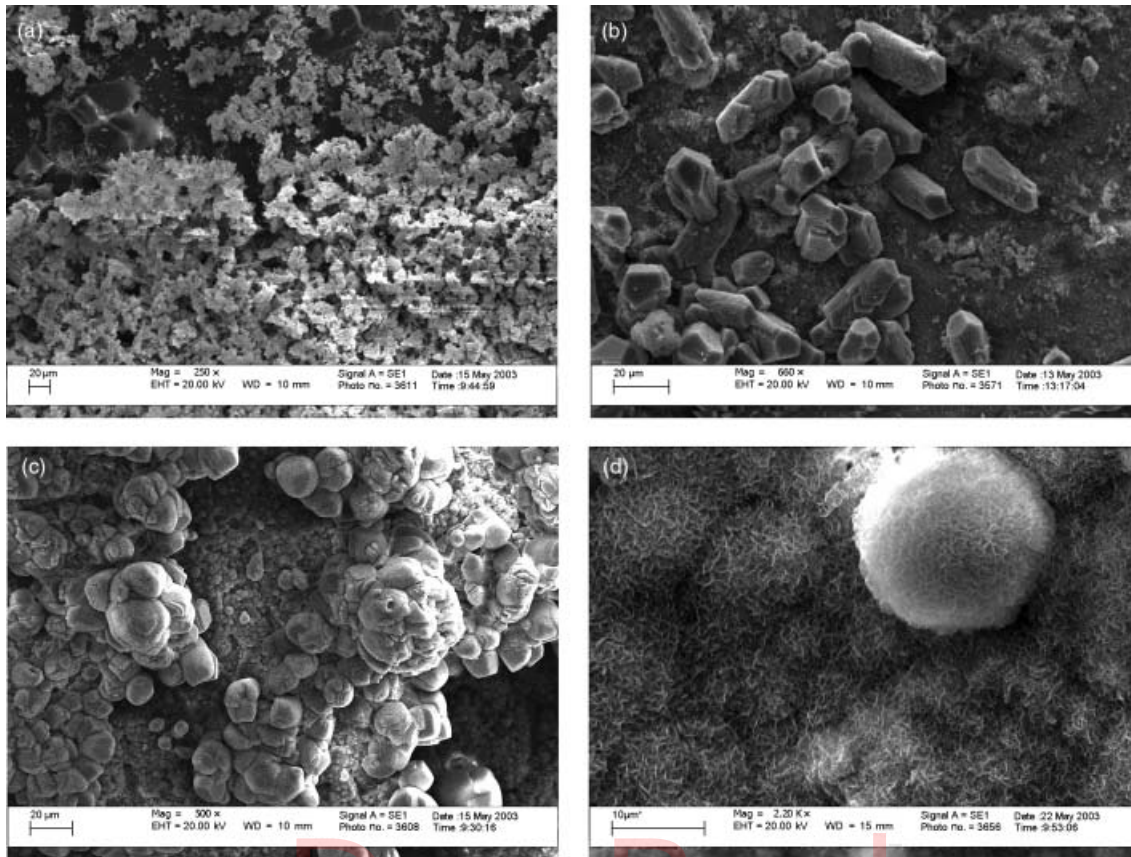


Figure 3 (a) Diffuse crystal deposition over cement surfaces (magnification $\times 250$). (b) Hexagonal crystal deposition over cement surfaces (magnification $\times 660$). (c) Granular material surface (magnification $\times 500$). (d) Coral-like appearance of material surface (magnification $\times 2,200$).

The critical-point drying method avoids these effects by never allowing a liquid/gas interface to develop.

The use of HMDS allowed better cell preservation and avoided the use of critical-point drying. However this technique still produced surface carbonation of the materials and the processing interfered with cell adhesion to the material. Increasing the cell incubation period might have allowed better cell adhesion. Only diffuse crystals (possibly calcites) were observed; the other polymorphs were not synthesized as the pressure and temperature were not modified. In view of these findings the current methodology for preparing specimens for SEM is not recommended for evaluation of the biocompatibility of MTA and materials with related chemistry due to processing artefacts produced by reactions occurring between the material and reagents used during the processing.

The cells were placed on the cements after being cured for two specific ages. By 1 day the primary

reaction, which allowed the material to set and harden was complete; by 28 days the hydration reaction was effectively complete. The two time intervals were selected to see if the reaction affected cell growth. In previous studies the materials were allowed to set for 1 week before cell contact (Mitchell *et al.* 1999, Abdullah *et al.* 2002).

The cell growth over 1-day cured MTA was better at the early stages than on the aged material. The primary reaction involving tricalcium silicate produced most of the calcium hydroxide (Lea 1970). The calcium hydroxide produced could be responsible for the early biocompatibility of the material. Thus, after incubating the cells for 5 days both white MTA and grey MTA showed cell confluence. This is in accordance with previous studies where cell confluence was observed over the surfaces of grey MTA after curing the material for 7 days (Mitchell *et al.* 1999, Abdullah *et al.* 2002). At 28 days of curing

the materials were less biocompatible and this may have been because less calcium hydroxide was available. The reduction in calcium hydroxide levels could possibly have been caused by surface carbonation of the material exposed to air during curing; however no carbonation was observed on the set cements cured for 28 days. An alternative explanation is that less calcium hydroxide was produced at 28 days as the tricalcium silicate reaction was complete; thus less calcium hydroxide was available to go into solution, and this could have caused the reduced biocompatibility of the MTA. After keeping the materials in solution for 7 days the increased amounts of calcium hydroxide could have encouraged cell proliferation.

There was no cell growth over bismuth oxide and alusilicate; this growth inhibition could have been due either to the surface roughness of the materials or because the chemical nature of the material did not encourage cell growth. The alusilicate would only be present in very small amounts in the white cement, but bismuth oxide is present in larger proportions. The lack of biocompatibility of the bismuth oxide did not seem to affect the biocompatibility of the MTA, presumably because of the presence of calcium hydroxide.

In view of previous cell culture studies (Mitchell et al. 1999, Abdullah et al. 2002) being indicative of tissue response (Torabinejad et al. 1997), the biocompatibility of white MTA in cell culture can be extrapolated to the likely response in tissue. It should be noted that the finding of biocompatibility of white MTA is in agreement with the results of Perez et al. (2003), who used a different transformed cell line. This study has suggested a possible mechanism for the biocompatibility of MTA.

Conclusions

The cells grown for 5 days over 1-day cured samples of the two commercial forms of MTA showed good biocompatibility. However, the 28-day cured samples were less biocompatible using this method as indicated by the lower scores.

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